Comparative antimicrobial activity and mechanism of action of bovine lactoferricin-derived synthetic peptides

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Abstract Lactoferricin B (LfcinB), a 25 residue peptide derived from the N-terminal of bovine lactoferrin (bLF), causes depolarization of the cytoplasmic membrane in susceptible bacteria. Its mechanism of action, however, still needs to be elucidated. In the present study, synthetic LfcinB (without a disulfide bridge) and LfcinB (C–C; with a disulfide bridge) as well as three derivatives with 15-, 11- and 9-residue peptides were prepared to investigate their antimicrobial nature and mechanisms. The antimicrobial properties were measured via minimum inhibitory concentration (MIC) determinations, killing kinetics assays and synergy testing, and hemolytic activities were assessed by hemoglobin release. Finally, the morphology of peptide-treated bacteria was determined by atomic force microscopy (AFM). We found that there was no difference in MICs between LfcinB and LfcinB (C–C). Among the derivatives, only LfcinB15 maintained nearly the same level as LfcinB, in the MIC range of 16–128 μg/ml, and the MICs of LfcinB11 (64–256 μg/ml) were 4 times more than LfcinB, while LfcinB9 exhibited the lowest antimicrobial activity.

When treated at MIC for 1 h, many blebs were formed and holes of various sizes appeared on the cell surface, but the cell still maintained its integrity. This suggested that LfcinB had a major permeability effect on the cytoplasmic membrane of both Gram-positive and Gram-negative bacteria, which also indicated it may be a possible intracellular target. Among the tested antibiotics, aureomycin increased the bactericidal activity of LfcinB against *E. coli*, *S. aureus* and *P. aeruginosa*, but neomycin did not have such an effect. We also found that the combination of cecropin A and LfcinB had synergistic effects against *E. coli*.

Introduction

Bovine lactoferricin (LfcinB), a 25-residue disulfide cross-linked peptide from the *N*-terminus of lactoferrin, possesses antimicrobial activities against a wide variety of microorganisms, including Gram-positive and Gram-negative bacteria, fungi and viruses (Bellamy et al. 1992; Orsi 2004; Gifford et al. 2005). Shorter derivatives of LfcinB, which were devoid of the disulfide bone, also exhibited antimicrobial activity (Ulvatne and Vorland 2001). These derivatives have been widely studied. A hexamer section (RRWQWR) of LfcinB displayed only weak activity (Nguyen et al.

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2010). However, when the negative charge at its *C*-terminus was neutralized by amidation, it was shown to display antimicrobial activity that compared favorably to LfcinB (Tomita et al. 1994; Schibli et al. 1999). An 11-residue linear derivative has also shown antimicrobial activity comparable to LfcinB (Nguyen et al. 2005) and a novel bovine lactoferrin peptide, FKCRR WQWRM, suppressed *Candida* cell growth efficiently (Ueta et al. 2001).

LfcinB has been shown to be more antimicrobial than any other lactoferricins, and its mode of action resembles that suggested for cecropins and magainins in that it disrupts the bacterial cell membrane (Isidra and Servaas 2000; Ulvatne et al. 2001). It has also been demonstrated that LfcinB, at concentrations not sufficient to kill bacterial cells, inhibits the incorporation of radioactive precursors into macromolecules in both Gram-positive and Gram-negative bacteria (Ulvatne et al. 2004) similar to buforin II (Park et al. 1998).

Several reports have shown that LfcinB possesses properties of synergy with different antibiotic substances such as erythromycin, minocycline and monoacylglycerol against *E. coli* and antibiotic-resistant strains of *S. aureus* (Vorland et al. 1999; Wakabayashi et al. 2002). This synergistic effect may be explained by either an enhanced uptake of the antibacterial substance or a sequential inhibition of a common pathway (Giacometti et al. 2000).

Although the antibacterial activity of LfcinB and its derived-peptides has been extensively documented, the mode of action of LfcinB is still not fully understood, and the mode of action has not yet been tested by AFM.

In the present study, we synthesized LfcinB (with and without a disulfide bridge) and its 15-, 11- and 9-residue derivatives to compare their antimicrobial activity. The morphology of peptide-treated *E. coli* and *S. aureus* was observed by AFM. Combinations of LfcinB with antibiotics were tested for synergistic effects by a checkerboard titration method. We further investigated the combined action of LfcinB and cecropinA to elucidate peptide-peptide interactions.

Materials and methods

Bacterial strains and growing conditions

The strains used were Gram-negative bacteria including *Escherichia coli* ATCC 25922, *Escherichia coli* K88, *Salmonella typhimurium* CMCC 50020, *Salmonella choleraesuis* CMCC 50013 and *Pseudomonas aeruginosa* CMCC 10014, as well as Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633, which were purchased from the China General Microbiological Culture Collection Center (Beijing, China). All strains were stored at −80°C, and further grown in Mueller–Hinton (MH) broth (Difco, England), pH 7.2, at 37°C. Antimicrobial activity tests were performed with cells in the exponential growth phase, and the cell suspension was adjusted to give a final density of $2–7 \times 10^5$ colony-forming units (CFUs)/ml.

Synthesis of peptides

The LfcinB-derived peptides used in this study were synthesized by GL Biochem Ltd. (Shanghai, China) using the solid-phase method (see Table 1 for detailed sequences). These peptides were purified by HPLC on a reversed-phase C_{18}-column. The purity level of each synthetic peptide was >95%, which was analyzed by peak integration with high-performing liquid chromatograms. A disulphide-bridge in LfcinB was formed by aerial oxidation of cysteinyl peptides. Figure 1 showed the HPLC (small box) and ESI–MS (large box) data of the LfcinB (C–C) and LfcinB. The peptides were dissolved in doubly distilled sterile water and stored at −80°C. Neomycin and aureomycin were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China).

Antimicrobial activity testing

The minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBSs) of the various lactoferricins were determined using an improved broth micro-dilution technique. Briefly, the MICs of the various LfcinB-derived peptides were determined in 2% MH broth after incubation at 37°C. The concentrations ranged from 4 to 256 µg/ml. The MIC was defined as the lowest concentration where no visible growth occurred. For determination of the MBC, aliquots of 10 µl were transferred onto agar plates. The plates were incubated overnight at 37°C, and the number of colony-forming units was determined. The MBC was set as the lowest concentration that reduced the number of CFUs by 99%. All assays were performed in triplicate at different times.

Table 1 Amino acid sequence of synthetic LFcinB and its derivatives

Lactoferricin	Location	Residues	MW calculated	Net charge	Hydrophobic ratio (%)	Amino acid sequence
LfcinB (C–C)	17–41	25	3123.8	8	48	FKCRRWQWRMKKLGAPSITCVRRAF
LfcinB	17–41	25	3125.8	8	48	FKCRRWQWRMKKLGAPSITCVRRAF
LfcinB15	17–31	15	1994.5	6	46	FKCRRWQWRMKKLGA
LfcinB11	20–30	11	1544.9	5	36	RRWQWRMKKLG
LfcinB9	20–28	9	1374.7	5	33	RRWQWRMKK

Hemolytic assay

The hemolytic activity of the synthesized peptides was evaluated by determining hemoglobin release from erythrocyte suspensions of fresh pig blood (Jinhua pigs, obtained from Zhejiang Jiahua Pig Breeding Co., Ltd). Blood was collected by venipuncture of an anterior vena cave in 5-ml vacutainer tubes (kangshi Co., China) containing sodium citrate as an anticoagulant. The cells were washed three times with phosphate-buffered saline (PBS, pH 7.2) and centrifuged at 1,500 rpm for 10 min at room temperature. The third supernatant liquid was clarified and colorless. Then the 0.1 ml erythrocyte suspension, diluted with 8.9 ml PBS (erythrocyte final concentration around 1%), was mixed with 0.1 ml of test substances at twofold serial concentrations (4–256 μg/ml). The mixtures were incubated at 37°C under 5% CO_2 for 18 h in microtiter plates. After incubation, plates were centrifuged at 1,500 rpm for 5 min. The supernatants were transferred into 96-well ELISA plates (cat. 3590, Costar, Cambridge, MA) and the optical density was measured at 414 and 546 nm using Multi-Mode Microplate Readers (SpectraMax M5, Molecular Devices, USA). The values for 0 and 100% lysis were determined by incubating erythrocytes with 10 mM PBS and 1% (v/v) Triton X-100, respectively. Three replicates were generated for each test solution.

Kinetics of bacterial killing

Killing by LfcinB (C–C) and LfcinB was investigated by testing E. coli ATCC 25922 and S. aureus ATCC 25923. The strains were grown at 37°C in MH broth. Aliquots of exponentially growing bacteria were resuspended in fresh MH broth at approximately $2–7 \times 10^5$ CFUs/ml and exposed to each peptide (final concentration $1 \times$ MIC, $3 \times$ MIC to E. coli

and $1/2 \times$ MIC, $1 \times$ MIC to S. aureus) for 0, 5, 10, 20, 30, 40, 50, 60, 90 and 120 min at 37°C. After these time periods, samples were serially diluted and plated onto MH agar plates to obtain viable colonies. Data are the mean of at least three independent determinations with comparable results.

AFM observation

A log phase culture of E. coli ATCC 25922 and S. aureus ATCC 25923 in MH broth were split into 1 ml aliquots. The cells were collected by centrifugation (4000 rpm, 10 min) and washed twice with PBS and resuspended in fresh MH broth. Two samples were prepared: one was untreated and the other was treated with LfcinB ($1 \times$ MIC). The samples were incubated at 37°C for 1 h with vigorous shaking. Then the cells were collected by centrifugation (4000 rpm, 10 min) to remove the MH broth and cells were resuspended in PBS. For AFM imaging in air, the samples were prepared by simply depositing 5 μl from suspensions of the control and peptide treated bacteria on the mica surface. After drying in ambient (25°C, 60% humidity) conditions, the sample was rinsed with deionized water for removal of salt crystals. AFM experiments in the tapping mode of operation were carried out using a SHIMADZU 9500J3 scanning probe microscope (SPM). Nanoprobe cantilevers made of lilicon nitride (Si3N4) with a spring constant of $k = 0.03$ N/m were used.

Synergy testing

The checkerboard titration method is widely used for evaluation of properties of different antibacterial combinations. An inoculum of logarithmic-phase bacteria ($2–7 \times 10^5$ CFU/ml) was cultured at 37°C for 12 h in each well of a 96-well flat-bottomed culture
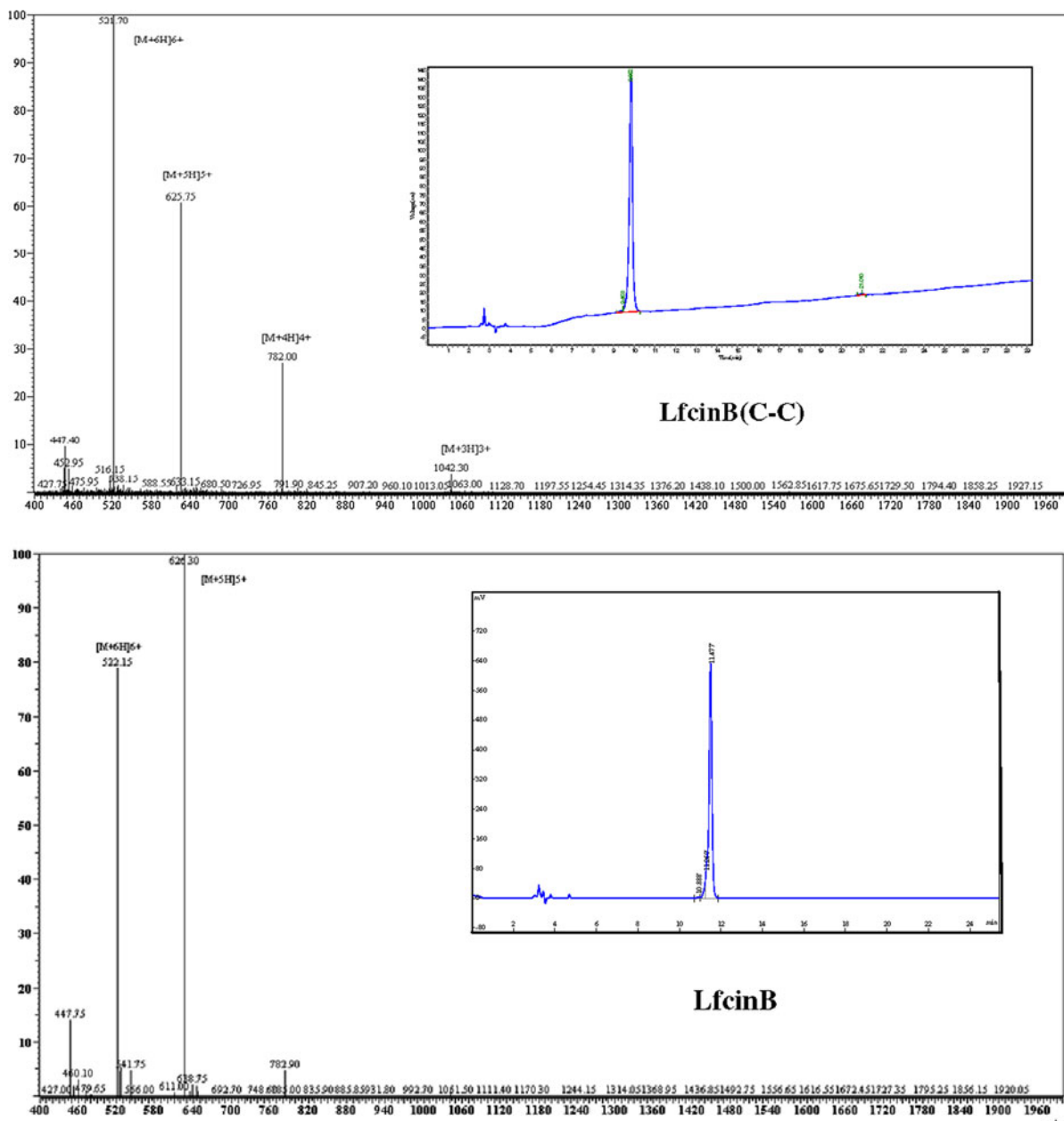
Fig. 1 HPLC and ESI–MS profiles of synthetic LfcinB25 (C–C) and LfcinB25. *Small box* HPLC (YMC A-302:ODS 150 × 4.6 mm ID, column temp 40 or 60°C, with 10–60% MeCN/water/0.1% TFA 20 min linear, 1.0 ml/min, detection at 220 nm), *large box* ESI–MS (RB-LM-01)

plate containing 100 µl of peptone broth with LfcinB or an antibiotic added, alone or in combination. The ranges of drug dilutions used were 0.125–256 µg/ml for LfcinB and Cecropin A and 0.0625–32 µg/ml for neomycin and aureomycin. The lowest concentration of each drug combination causing growth inhibition or with bactericidal activity was plotted on an arithmetic

scale and synergy was analyzed from the shape of the curve and the fractional inhibitory concentration (FIC) index. The FIC index was calculated as follows: FIC index $= [(A)/MIC_A] + [(B)/MIC_B]$, where MIC_A and MIC_B are the MICs of drugs A and B alone, respectively, and A and B are the MICs of drugs A and B in combination, respectively. Trials were performed

at least three times with each antimicrobial combination to ensure reproducibility.

Results

### Antimicrobial activity of LfcinB-derived peptides

The MIC values of LfcinB and its derivatives for *E. coli*, *S. typhimurium*, *S. choleraesuis* and *P. aeruginosa*, *S. aureus* and *B. subtilis* are shown in Table 2. We found that there was almost no difference in MICs between LfcinB and LfcinB (C–C). Only LfcinB15 maintained the same level as LfcinB, in the MIC range of 16–128 µg/ml, and the MICs (64–256 µg/ml) of LfcinB11 were 4 times more than LfcinB. Moreover, LfcinB9 exhibited much lower antimicrobial activity against Gram-negative and Gram-positive bacteria (MIC and MBC values ranged from 256 to >256 µg/ml).

Hemolytic activity

The toxicity for eukaryotic cells of LfcinB-derived peptides was determined as the ability to lyse pig erythrocytes (Fig. 2). LfcinB (C–C) and LfcinB displayed a slight hemolytic activity at 64 µg/ml, at which concentration they exhibited a marked antimicrobial activity against most bacteria tested. However, LfcinB (C–C) showed a slightly lower hemolytic activity than LfcinB, but only 256 µg/ml and 64 µg/ml demonstrated significant ($P < 0.05$) hemolytic activity. Among these peptides, LfcinB9 displayed the lowest hemolytic activity even though it showed no antimicrobial activity at a concentration of 256 µg/ml.

Kinetics of bacterial killing

The activity of peptides on the motility of two representative isolates, *E. coli* and *S. aureus,* was both dose- and time-dependent. The results are summarized in Fig. 3, which shows typical curves for LfcinB and LfcinB (C–C). The higher the concentration used, the faster the activity exerted by synthetic peptides against bacteria. In particular, at a concentration equal to the MIC, they caused at least a 99% viability reduction of *S. aureus* cells within 10 min of incubation, while the killing of *E. coli* cells appeared somewhat less rapid. The antibacterial efficiency of LfcinB (C–C) and LfcinB showed no difference. At $1 \times$ MIC, complete killing of *E. coli* and *S. aureus* was observed after 40 min and 10 min, respectively. A sub-minimal inhibitory concentration (8 µg/ml) of these two
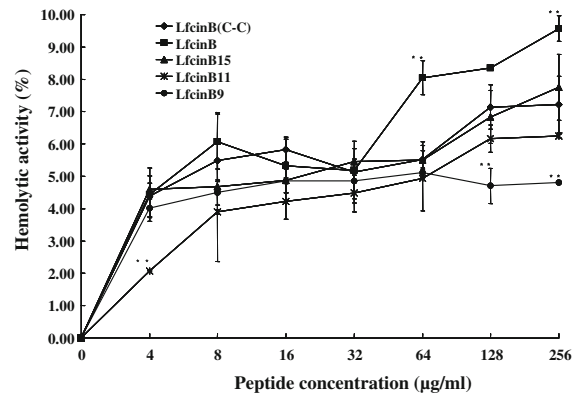
Fig. 2 Hemolytic activity of bovine lactoferricin-derived synthetic peptides against pig erythrocytes. Hemolysis was determined by measuring hemoglobin absorbance in the supernatant presented as percentage hemolysis achieved with 0.1% Triton-X-100. The results represent the mean ± standard deviation of at least four independent experiments

Table 2 MICs of lactoferricins against Gram-negative and Gram-positive bacteria

Peptides	MIC/MBC (µg/ml)						
	E.coli ATCC 25922	*E. coli* K 88	*S. choleraesuis* CMCC50020	*S. typhimurium* CMCC50013	*P. aeruginosa* CMCC27853	*S. aureus* ATCC 25923	*B. subtilis* ATCC6633
LfcinB (C–C)	16/32	64/128	32/64	32/64	128/256	16/32	8/16
LfcinB	32/64	64/128	32/64	32/64	128/256	16/32	8/16
LfcinB15	32/64	64/128	32/128	32/64	256/NF	32/128	32/128
LfcinB11	32/128	64/128	128/256	64/256	256/NF	128/NF	8/16
LfcinB9	256/NF	NF/NF	NF/NF	NF/NF	NF/NF	NF/NF	NF/NF
Aureomycin	4/16	2/8	4/16	4/16	8/8	0.06/0.25	2/8
Neomycin	2/8	2/8	2/8	2/8	8/16	0.13/0.5	1/4

peptides suppressed the growth of *S. aureus* within 30 min. It is worth noting that they inactivated target cells more quickly and effectively than most currently used antibiotics. For example, aureomycin, when tested against *E. coli* and *S. aureus*, does not reach the bactericidal endpoint (99.9% killing) at its MIC value within a 2 h incubation.
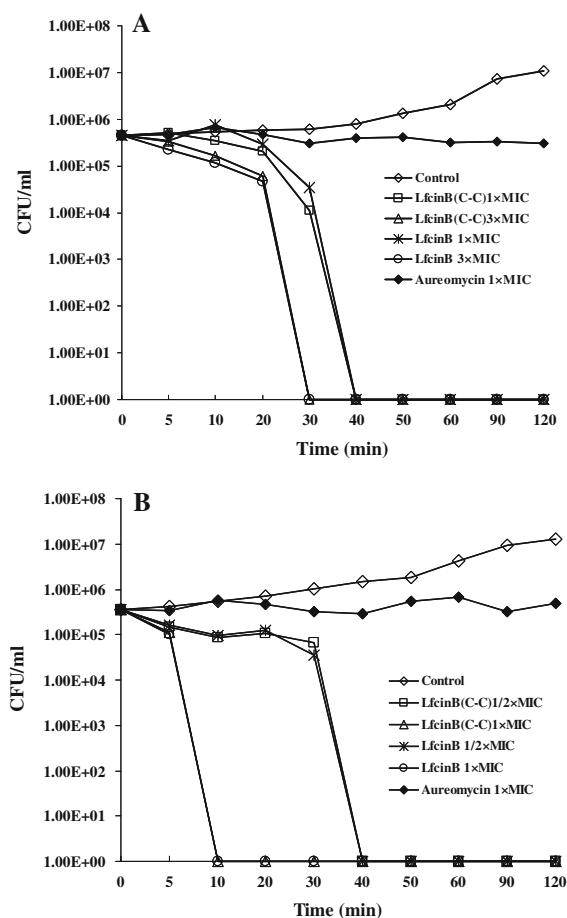
AFM observations

Escherichia coli and *S. aureus* cells were treated with LfcinB at a concentration of $1\times$ MIC before they were dried at room temperature on a mica surface and imaged using AFM. For each sample, duplicate slides

Fig. 3 Killing kinetics of LfcinB (c–c) and LfcinB25 against *E. coli* (**a**) and *S. aureus* (**b**). *E. coli* and *S. aureus* cells were incubated with the indicated concentrations of the peptides in MHB medium. At the indicated times, survivors were diluted in double distilled water and plated to allow colony counts. Results are mean values of at least four independent determinations

were made and numerous randomly chosen parts of each slide were imaged. The images shown are typical. In these images the light areas are parts of the sample that were high and the dark areas are parts of the sample that were low.

The AFM images of freshly prepared untreated *E. coli* and *S. aureus* (Fig. 4a, d) showed the surface roughness. After 1 h at $1 \times$ MIC concentration of LfcinB, the initial morphological changes observed were numerous black hole-like structures appearing on the surface of some cells as well as some indentations. This perturbation may have damaged the outer membrane, thus exposing the peptidoglycan wall beneath it. After treatment for 3 h, there were a lot fewer whole cells present on the slide, and the randomly chosen areas for imaging contained less and, in some cases, no cells (data not shown). This indicated that LfcinB may have weakened the cell walls and caused the cells to be leaky, which made them prone to lysis.

Interaction of LfcinB with antibiotics and other peptides

The FIC indexes for combinations between LfcinB and aureomycin, neomycin, and cecropin A are summarized in Table 3. From the results obtained, it appears that a synergistic effect was obtained between LfcinB and aureomycin and cecropin A, except for *S. aureus* with cecropin A based on its high MIC. No synergy, but independent action, was found in all bacteria tested when combining LfcinB and neomycin. No antagonism was observed between the antibacterial agents used and LfcinB against *E. coli*, *S. aureus* and *P. aeruginosa*. The FIC indexes ranged from 0.375 to 0.5 for aureomycin and cecropin A. The MICs of the antibiotics decreased obviously while the peptide was added (data not shown), indicating that LfcinB could act in combination with these antimicrobial agents against *E. coli*, *S. aureus* and *P. aeruginosa*.

Discussion

We used a set of peptides of different lengths to further understand the mode of action of LfcinB, a 25-residue peptide that forms into a looped structure through an intramolecular disulfide bond. We have comprehensively investigated their antibacterial and cytotoxic activities, the mechanism of action towards

Fig. 4 The AFM images of freshly untreated *E. coli* (**a**) and *S. aureus* (**c**; *the left panels* are deflection images and the right panels are 3D reconstructions). **b** *E. coli* treated with 32 μg/ml of LfcinB25 for 30 min. **d** *S. aureus* treated with 32 μg/ml of LfcinB25 for 30 min
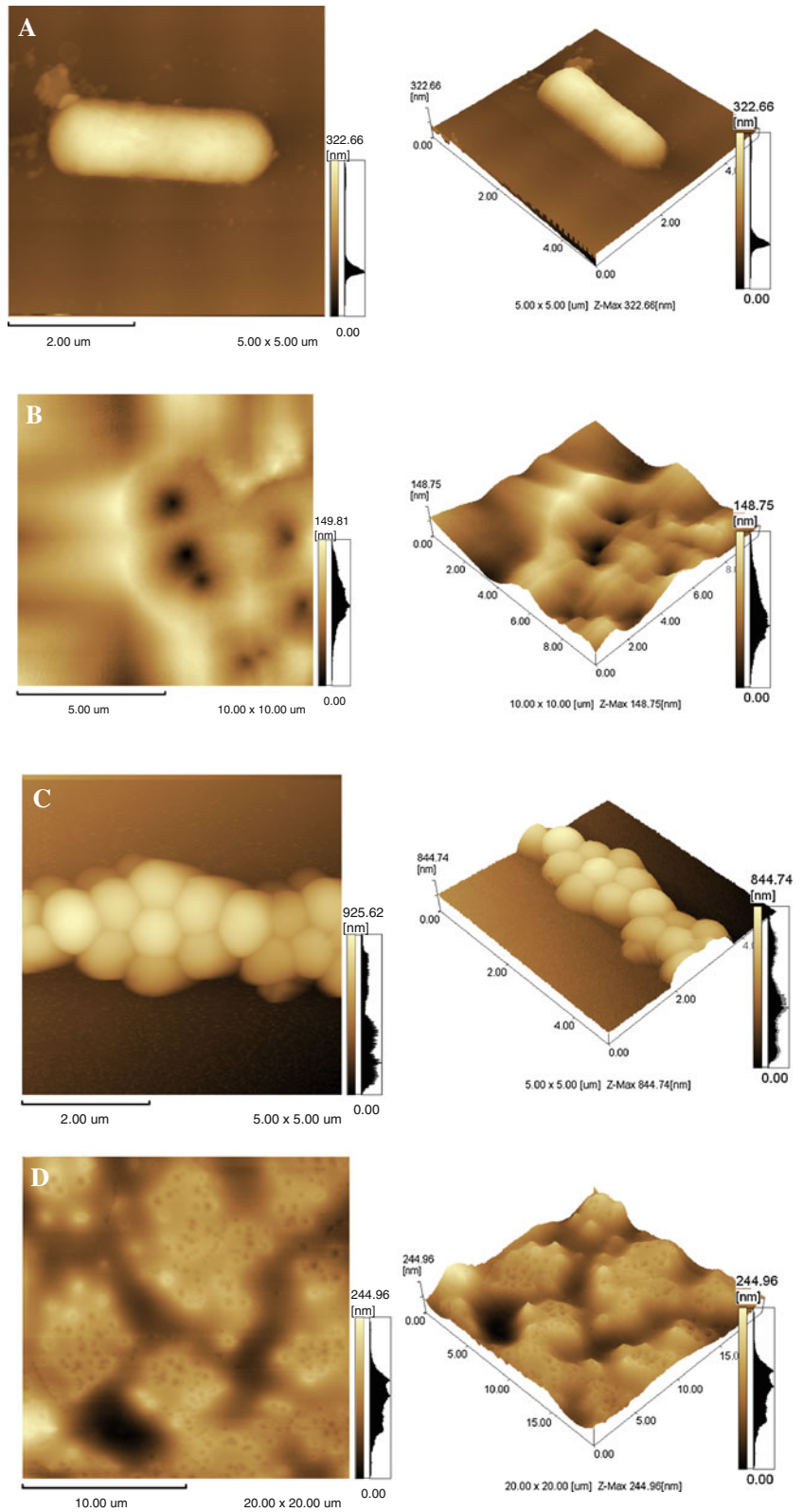
Table 3 The FIC indexes[a] for LfcinB25 in combination with conventional antibiotics and CecropinA against *E. coli*, *S. aureus* and *P. aeruginosa*[b]

Organism	Aureomycin	Neomycin	Cecropin A
E. coli	0.375	1.25	0.5
S. aureus	0.5	1.5	–
P. aeruginosa	0.5	1.25	0.5

[a] Synergic action is defined as FIC ≤ 0.5, partial synergism as $0.5 <$ FIC ≤ 1, indifference $1 <$ FIC ≤ 4, and antagonistic action as FIC > 4

[b] The ranges of concentrations tested were 0.5–256 µg/ml for LfcinB25 and Cecropin A and 0.0625–32 µg/ml for the other antimicrobial compounds

E. coli and *S. aureus* by AFM, and synergic interactions between LfcinB and conventional feed antibiotics and ceceopin A.

NMR spectroscopy studies indicated that LfcinB adopts a twisted β-sheet in low-salt solutions, which is quite different from the α-helix seen in intact bLF (Hwang et al. 1998). Some antibacterial peptides are clearly multifunctional and an attempt to predict possessing positive net charge and amphipathicity with a hinge that will help the molecule to 'flip' into a bacterial membrane (Boman 2003). LfcinB, with a +8 net charge, has an extended hydrophobic surface and its total hydrophobic ratio can be up to 48% (Gifford et al. 2005). By adopting this structure, LfcinB becomes markedly amphipathic with clear hydrophobic and positively charged faces, which is a trait they share with other peptides that display antimicrobial activity. Previous studies demonstrated that shorter LfcinB-related peptides with N-acylated or D enantiomer properties showed antimicrobial activities greater than LfcinB against bacteria and fungi (Wakabayashi et al. 1999). In our study, Lfcin9 did not show any antimicrobial activities and Lfcin11 had four to eight times higher MIC than LfcinB, which may be related to the absence of N-acylation. However, we found that LfcinB15, with higher net charge and a larger amount of hydrophobic residues than LfcinB11 and LfcinB9, experienced only a minor loss of antimicrobial activity relative to the native peptide, which was in the MIC range of 32–256 µg/ml. To gain insight into the structural properties of LfcinB, comparison study between LfcinB without a disulphide bridge and LfcinB (C–C) with a disulphide bridge was performed. It is reported that peptides corresponding to parts of LFcin without a disulfide bridge also showed killing of

representative species, including *C. albicans* and *E. coli* (Groenink et al. 1999; Van der Kraan et al. 2004). However, this result was later contradicted by Jenssen et al. (2004) who suggested that the deficiency of this loop structure could loosen or weaken the antibiotic activity. The present investigation indicated that there was little activity lost by the reduction of the disulfide bridge for LfcinB, however, the hemolytic activity was slightly raised. Recently, several studies have focused on the immunomodulatory effects and anticancer activity of LfcinB and it appears that deficiencies in the loop structure can cause LfcinB to lose the biological activity of inducing apoptosis in Jurkat cells (Zhang et al. 2010) and do not exhibit a cytotoxic effect to neuroblastoma cells (Eliassen et al. 2006).

From the point of view of selecting a peptide as a potential antimicrobial agent, strong interactions with bacterial membranes is encouraging. Conversely, it is required to find peptides with relatively weak binding with mammalian membranes (Tolokh et al. 2009). LfcinB and some derived peptides have high bactericidal activities that could be attributed to pore formation in the susceptible bacterial membrane (Umeyama et al. 2006). However, for *E. coli* and *S. aureus,* a cytoplasmic target has also been suggested (Haukland et al. 2001). In the present work, the bactericidal effect of LfcinB upon *E. coli* and *S. aureus* was analyzed through AFM. AFM is a powerful imaging tool that is capable of achieving high resolution images of biological samples even under physiological conditions, such as in fluid and at physiological temperature. The use of AFM has provided vivid and detailed close-up images of the bacteria undergoing various stages of antimicrobial peptide actions on a nanometer scale (Li et al. 2007). We have shown that LfcinB at MIC induced extensive permeation and collapse of *E. coli* and *S. aureus* (Fig. 4). The same result was obtained with LfcinB (C–C; data not shown). A direct inhibitory effect of LfcinB on bacterial protein-synthesis was also demonstrated according to the cell-free protein synthesis assay (data not shown), which was similar to that observed previously (Ulvatne et al. 2004). In our study, LfcinB did not destroy the integrity of the *E. coli* and *S. aureus* membranes (Fig. 4). These observations indicated that LfcinB exerts its bactericidal effect by acting on the bacterial cell surface initially, and then on the cytoplasmic contents.

Based on the mechanism suggested above, LfcinB can change outer membrane structure and cause loss of inner membrane integrity, and it can promote the uptake of other agents; e.g., antibiotics and other antimicrobial peptides, thus showing synergy with conventional antibiotics, especially against antibiotic resistant mutants (Hancock and Sahl 2006). As shown in Table 3, LfcinB was synergistic with aureomycin in all tested bacteria. In addition, a strong synergy between LfcinB and cecropin A was also observed against *E. colli* and *P. aeruginosa.* However, there is only an independent effect between LfcinB and neomycin. Thus, the remaining question is to explain why synergistic effects of LfcinB were only observed with some classes of antimicrobial agents instead of with all classes of antimicrobial agents. Aminoglycosides exhibit a rapid lethal effect, and it is now recognized that they competitively displace cell biofilm-associated Mg^{2+} and Ca^{2+}. This mechanism is much the same as described for the cationic peptides, including LfcinB. The expected effect of LfcinB on the cytoplasmic membrane should antagonize the effect of neomycin on the ribosomes by preventing it reaching its presumed target.

Conclusion

Although some in vivo trials have been performed with LfcinB, most studies to date have only evaluated the in vitro activity of these antibacterial components. As to the future, an important development will be offered by the potential prophylactic and therapeutic use of LfcinB or its derived peptides in a wide spectrum of medical conditions. An interesting perspective that can be foreseen is the use of LfcinB or its derived peptides in combination with other antimicrobial compounds.

References

Bellamy W, Takase M, Wakabayashi H et al (1992) Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. J Appl Bacteriol 73(6):472–479

Boman HG (2003) Antibacterial peptides: basic facts and emerging concepts. J Intern Med 254(3):197–215

Eliassen LT, Berge G, Leknessund A et al (2006) The antimicrobial peptide, lactoferricin B, is cytotoxic to neuroblastoma cells in vitro and inhibits xenograft growth in vivo. Int J Cancer 119(3):493–500

Giacometti A, Cirioni O, Prete MS et al (2000) Combination studies between polycationic peptides and clinically used antibiotics against Gram-positive and Gram-negative bacteria. Peptides 21(8):1155–1160

Gifford JL, Hunter HN, Vogel HJ (2005) Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. Cell Mol Life Sci 62(22):2588–2598

Groenink J, Walgreen-Weterings E, van't Hof W et al (1999) Cationic amphipathic peptides, derived from bovine and human lactoferrins, with antimicrobial activity against oral pathogens. FEMS Microbiol Lett 179(2):217–222

Hancock RE, Sahl HG (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 24(12):1551–1557

Haukland HH, Ulvatne H, Sandvik K et al (2001) The antimicrobial peptides lactoferricin B and magainin 2 cross over the bacterial cytoplasmic membrane and reside in the cytoplasm. FEBS Lett 208(3):389–393

Hwang PM, Zhou N, Shan X et al (1998) Three-dimensional solution structure of lactoferricin B, an antimicrobial peptide derived from bovine lactoferrin. Biochemistry 37(12):4288–4298

Isidra Recio, Servaas Visser (2000) Antibacterial and binding characteristics of bovine, ovine and caprine lactoferricins: a comparative study. Int Dairy J 10(9):597–605

Jenssen H, Andersen JH, Uhlin-Hansen L et al (2004) Anti-HSV activity of lactoferricin analogues is only partly related to their affinity for heparin sulfate. Antivir Res 61(2):101–109

Li A, Lee PY, Ho B et al (2007) Atomic force microscopy study of the antimicrobial action of Sushi peptides on Gram negative bacteria. Biochim Biophys Acta 1768(3):411–418

Nguyen LT, Schibli DJ, Vogel HJ (2005) Structural studies and model membrane interactions of two peptides derived from bovine lactoferricin. J Peptide Sci 11(7):379–389

Nguyen LT, Chau JK, Perry NA et al (2010) Serum stabilities of short tryptophan- and arginine- rich antimicrobial peptide analogs. PLoS One 5(9):e12684

Orsi N (2004) The antimicrobial activity of lactoferrin: current status and perspectives. Biometals 17(3):189–196

Park CB, Kim HS, Kim SC (1998) Mechanism of action of the antimicrobial peptide buforin II: bufrrinII kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. Biochem Biophys Res Commun 244(1):253–257

Schibli DJ, Hwang PM, Vogel HJ (1999) The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles. FEBS Lett 446(2–3):213–217

Tolokh IS, Vivcharuk V, Tomberli B, et al (2009) Binding free energy and counterion release for adsorption of the antimicrobial peptide lactoferricin B on a POPG membrane. Phys Rev E Stat Nonlin Soft Matter phys 80(3 Pt 1): 031911

Tomita M, Takase M, Bellamy W et al (1994) A review: the active peptide of lactoferrin. Acta Paediatr Jpn 36(5):585–591

Ueta E, Tanida T, Osaki T (2001) A novel bovine lactoferrin peptide, FKCRRWQWRM, suppresses Candida cell growth and activates neutrophils. J Peptide Res 57(3):240–249

Ulvatne H, Vorland LH (2001) Bactericidal kinetics of 3 lactoferricins against *Staphylococcus aureus* and *Escherichia coli*. Scand J Infect Dis 33(7):507–511

Ulvatne H, Haukland HH, Olsvik Ø, Vorland LH (2001) Lactoferricin B causes depolarization of the cytoplasmic membrane of *Escherichia coli* ATCC 25922 and fusion of negatively charged liposomes. FEBS Lett 492(1–2):62–65

Ulvatne H, Samuelsen Ø, Haukland HH et al (2004) Lactoferricin B inhibits bacterial macromolecular synthesis in *Escherichia coli* and *Bacillus subtilis*. FEMS Microbiol Lett 237(2):377–384

Umeyama M, Atsushi Kira, Nishimura K et al (2006) Interactions of bovine lactoferricin with acidic phospholipids bilayers and its antimicrobial activity as studied by solid-state NMR Biochim. Biophys Acta 1758(9):1523–1528

Van der Kraan Mi, Groenink J, Nazmi K et al (2004) Lactoferrampin: a novel antimicrobial peptide in the N1-domain of bovine lactoferrin. Peptides 25(2):177–183

Vorland LH, Osbakk SA, Perstølen T et al (1999) Interference of the antimicrobial peptide lactoferricin B with the action of various antibiotics against *E coli* and *S aureus*. Scand J Infect Dis 31(2):173–177

Wakabayashi H, Matsumoto H, Hashimoto K et al (1999) *N*-Acylated and D enantiomer derivatives of a nonamer core peptide of lactoferricin B showing improved antimicrobial activity. Antimicrob Agents Chemother 43(5):1267–1269

Wakabayashi H, Teraguchi S, Tamura Y (2002) Increased Staphylococcus-killing activity of an antimicrobial peptide, Lactoferricin B, with minocycline and monoacylglycerol. Biosci Biotechnol Biochem 66(10):2161–2167

Zhang TN, Yang W, Liu N (2010) Effect of loop structure of bovine lactoferricin on apoptosis in Jurkat cells. Biometals 23(3):555–561